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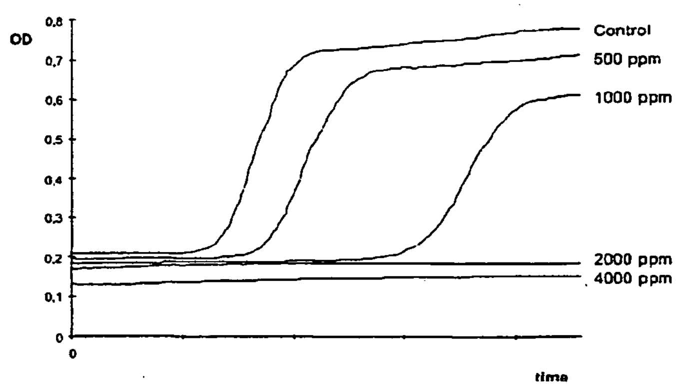
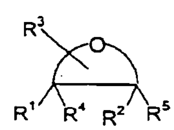
(54) Title: ANTIMICROBIAL AGENT





(I)

(57) Abstract: The present invention provides an antimicrobial composition for use against a micro-organism selected for *Listeria, Salmonella, Bacillus, Saccharomyces, Pseudomonas, Clostridium, Lactobacillus, Brochothrix, Micrococcus, Yersinia, Enterobacter* and *Zygosaccharomyces*, said composition comprising a cyclic compound having Formula (I), or a derivative thereof, wherein R^1 and R^2 are independently selected from -OH, =O, and OR', wherein R' is H or -COR'', and R'' is C_{1-10} alkyl; wherein R^3 is a substituent comprising an -OH group; wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound. The invention further relates to a process for preventing and/or inhibiting the growth of, and/or killing, micro-organisms in a material, and the use of a cyclic compound having Formula (I).

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ANTIMICROBIAL AGENT

The present invention relates to antimicrobial agents. More specifically, the invention relates to the antimicrobial activity of a series of anhydrofructose derivatives.

Food degradation from various sources is recognised in the literature and individual chemicals are known which will inhibit one aspect or another of degradation derived from a single source. Degradation, and the loss of colour or flavour of freshly cut plant parts are known to be caused by oxidation, enzymes, microbes, and metal ions. For example, acidulants are known to prevent microbial degradation by maintaining a relatively low pH environment but their effectiveness is only temporary.

Salmonella, of which there are over two thousand different strains, is one of the major causes of food poisoning in humans. *Salmonella* is a genus of rod-shaped Gram-negative *Enterobacteriaceae* that inhabit the intestine and cause infections such as gastroenteritis and typhoid. If invasive, they can cause enteric fevers (for example, typhoid caused by *Salmonella typhi,* or paratyphoid fever caused by *Salmonella paratyphi*). Other strains of *Salmonella* are associated with food poisoning (usually *Salmonella* Typhimurium, *Salmonella panama* or *Salmonella* Enteritidis, the latter notorious for the contamination of poultry) and occasionally septicaemia in non-intestinal tissues.

It is well known in the art that *Salmonella* cannot propagate at pH values below 4.5. As a consequence, mildly acid products such as fine food and non-fermented meat products are especially susceptible to attack by *Salmonella.*

For meat products, nitrite is often used as a preservative. However, the addition of nitrite is restricted for toxicological reasons (due to its acute toxicity, together with the dangers associated with nitrosamine formation). As a result, *Salmonella* is only inhibited at concentrations of nitrite beyond 1,000 ppm, which are far beyond legal limits.

Instead, it has been shown that combinations of nitrite and sorbic acid can increase the effectiveness against *Salmonella* [Inhibition of *Salmonella* by Sodium Nitrite and

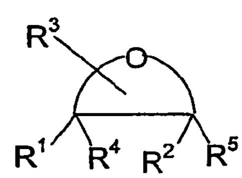
Potassium Sorbate in Frankfurters, Journal of Food Science, 47, 1982, p. 1615 ff]. Inhibition has been observed at concentrations beyond 50 ppm of nitrite combined with 2600 ppm sorbic acid.

Other agents such as bacteriocins (Nisin) are unable to inhibit *Salmonella* in food, whereas benzoic acid is unsuitable because the inhibitory effect can only be observed in acid products. The inhibitory effect of phytogenic ingredients (or "natural substances") such as oil extracts from different spices, has also been tested, but again the concentrations required for achieving the inhibitory effect on *Salmonella* were too high and the sensorical influence on the food was too strong.

Thus, to date, the use of chemical substances has been severely limited because on the one hand they have to be safe from a toxicological view point, but on the other hand they must not influence the product sensorically.

The present invention seeks to alleviate the problems associated with prior art chemical substances and to provide new antimicrobial compositions based on anhydrofructose derivatives. In particular, the invention seeks to provide antimicrobial agents that are suitable for use in foodstuffs/feed.

In a first aspect, the invention provides an antimicrobial composition for use against a micro-organism selected from *Listeria, Salmonella, Bacillus, Saccharomyces, Pseudomonas, Clostridium, Lactobacillus, Brochothrix, Micrococcus, Yersinia, Enterobacter* and *Zygosaccharomyces,* said composition comprising a cyclic compound having Formula I,



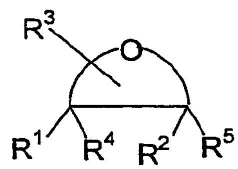
I

or a derivative thereof,

wherein R^1 and R^2 are independently selected from -OH, =O, and OR', wherein R' is H or -COR", and R" is C_{1-10} alkyl;

wherein R^3 is a substituent comprising an -OH group;
wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound.

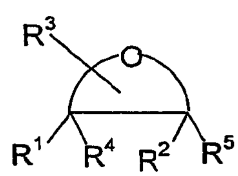
In a second aspect, the invention provides a process for preventing and/or inhibiting the growth of, and/or killing, micro-organisms in a material, the process comprising the step of contacting the material with a cyclic compound having Formula I,



I

or a derivative thereof,
wherein R^1 and R^2 are independently selected from -OH, =O, and OR', wherein R' is H or -COR", and R" is C_{1-10} alkyl;
wherein R^3 is a substituent comprising an -OH group;
wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound;
wherein said micro-organism is selected from *Listeria, Salmonella, Bacillus, Saccharomyces, Pseudomonas, Clostridium, Lactobacillus, Brochothrix, Micrococcus, Yersinia, Enterobacter* and *Zygosaccharomyces.*

In a third aspect, the invention relates to the use of a compound having Formula I, or a derivative thereof,



I

wherein R^1 and R^2 are independently selected from -OH, =O, and OR', wherein R' is H or -COR", and R" is C_{1-10} alkyl;
wherein R^3 is a substituent comprising an -OH group;

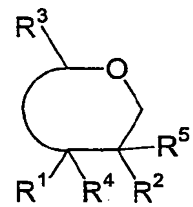
wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound;
for preventing and/or inhibiting the growth of, and/or killing, micro-organisms in a material, wherein said micro-organism is selected from *Listeria, Salmonella, Bacillus, Saccharomyces, Pseudomonas, Clostridium, Lactobacillus, Brochothrix, Micrococcus, Yersinia, Enterobacter* and *Zygosaccharomyces.*

Preferably, the material is a foodstuff or feed. Thus, in a preferred aspect, the present invention relates to antimicrobial substances that are suitable for use in foodstuffs and/or feed to inhibit food poisoning and spoiling bacteria contained therein.

By way of definition, the term "antimicrobial" refers to a substance that kills or prevents or inhibits the growth or reproduction of micro-organisms. Antimicrobials are generally classified according to the type of micro-organism they are effective against. For example, antibacterial substances are effective against bacteria, antifungal substances are effective against fungi, including yeast, and antiviral substances are effective against viruses. Certain antimicrobials can be used internally, for example antibiotic medications, whereas other antimicrobials are for external use only, such as antiseptics.

In a preferred aspect, the cyclic compound of the invention is a compound having formula I, or a derivative thereof,
wherein R^1 and R^2 are independently selected from -OH, =O;
wherein R^3 is a substituent comprising an -OH group;
wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound.

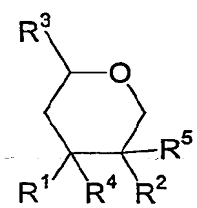
In a more preferred aspect, the cyclic compound of the invention is a compound having Formula II



II

or a derivative thereof, wherein R^1, R^2, R^3, R^4, and R^5 are as defined above.

In a further preferred aspect, the cyclic compound of the invention is a compound having Formula III



III

or a derivative thereof; wherein R^1, R^2, R^3, R^4, and R^5 are as defined above.

In one preferred aspect of the invention the groups R^4 and R^5 of the general formula may independently be a hydrocarbyl group.

The term "hydrocarbyl group" as used herein means a group comprising at least C and H and may optionally comprise one or more other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, hydroxy, carboxyl, epoxy, acrylic, hydrocarbon, N-acyl, or cyclic group etc. In addition to the possibility of the substituents being a cyclic group, a combination of substituents may form a cyclic group. If the hydrocarbyl group comprises more than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked *via* a suitable element or group. Thus, the hydrocarbyl group may contain hetero atoms. Suitable hetero atoms will be apparent to those skilled in the art and include, for instance, sulphur, nitrogen and oxygen.

The groups R^4 and R^5 of the general formula may independently be selected from alkyl, alkenyl, cycloalkyl and aryl or may together represent an alkylene.

In a particularly preferred aspect of the invention, the derivative of the compound of Formula I is an ester. The term "ester" includes mono-, di-, tri- and poly-esters.

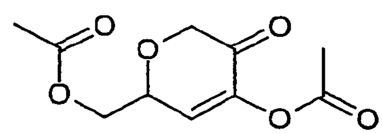
Preferably, the derivative of the compound of formula I is an ester wherein an ester linkage is formed from the -OH group of the R^3 substituent. In this aspect preferably the derivatised R^3 substituent is a group of the formula $-(CH_2)_n-OC(O)-(CH_2)_pCH_3$, wherein n and p are independently of each other from 1 to 24, preferably from 1 to 20, preferably from 1 to 10, preferably from 1 to 5, or preferably 1, 2, or 3. In yet a further preferred embodiment the derivatised R^3 substituent is a group of the formula $-CH_2-OC(O)-(CH_2)_pCH_3$, wherein p is from 1 to 24, preferably from 1 to 20, or p is from 1 to 10, or p is from 1 to 5, and n=1, 2, or 3.

Preferably, the derivative of the compound of formula I is an ester wherein the R^1 substituent and/or the R^2 substituent is an -OH group and wherein an ester linkage is formed from the -OH group of the R^1 substituent and/or the R^2 substituent. In this aspect preferably the derivatised R^1 substituent and/or the R^2 substituent is a group of the formula $-(CH_2)_n-OC(O)-(CH_2)_pCH_3$, wherein n and p are independently of each other from 1 to 24, preferably from 1 to 20, preferably from 1 to 10, preferably from 1 to 5, or preferably 1, 2, or 3. In yet a further preferred embodiment the derivatised R^1 substituent and/or the R^2 substituent is a group of the formula $-CH_2-OC(O)-(CH_2)_pCH_3$, wherein p is from 1 to 24, preferably from 1 to 20, or p is from 1 to 10, or p is from 1 to 5, and n=1, 2, or 3.

Preferably, the derivative of the compound of formula I is an ester wherein the R^1 substituent and/or the R^2 substituent is an -OH group and wherein an ester linkage is formed from the -OH group of the R^1 substituent and/or the R^2 substituent. In this aspect preferably the derivatised R^1 substituent and/or the R^2 substituent is a group of the formula $-OC(O)-(CH_2)_pCH_3$, wherein p is from 1 to 24, preferably from 1 to 20, preferably from 1 to 10, preferably from 1 to 5, or preferably 1, 2, or 3.

In a preferred aspect the compound of formula I is a diester wherein the R^1 substituent is an -OH group and wherein the ester linkages are formed from the -OH group of the R^4 substituent and from the -OH group of the R^3 substituent.

In a highly preferred aspect a derivative of the compound of formula I is a compound of the formula

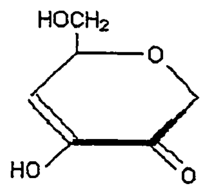


This compound (3,6-di-*O*-acetyl-1,5-anhydro-4-deoxy-D-*glycero*-hex-3-enopyranose-2-ulose) may be prepared in accordance with the teaching of Andersen *et al.* (1998), Structure of 1,5-anhydro-D-fructose: X-ray analysis of crystalline acetylated dimeric forms, J. Carbohydr. Chem. 17: 1027-1035.

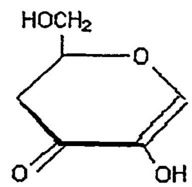
The aspect of the present invention wherein the derivative of the compound of formula I is an ester is particularly preferred because the compound may be lipophilic and/or may have both hydrophobic and hydrophilic properties. When the compound has both hydrophobic and hydrophilic properties the compound readily resides at a water/oil interface of an emulsion.

The residence of the compound at a water/oil interface of an emulsion may allow it to act as an emulsifier. Thus the present invention may further provide compounds having a dual functional effect. The compounds may act both as an antimicrobial and as an emulsifier.

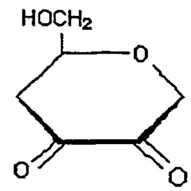
In particularly preferred aspect of the invention, the cyclic compound is selected from Ascopyrone P, Ascopyrone M, Ascopyrone T, Ascopyrone T_1, Ascopyrone T_2, and Ascopyrone T_3, and mixtures thereof, the structures of which are shown below.



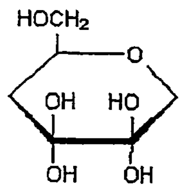
Ascopyrone M



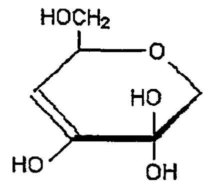
Ascopyrone P



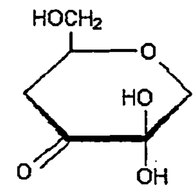
Ascopyrone T



Ascopyrone T_1



Ascopyrone T_2



Ascopyrone T_3

Ascopyrone is a known compound. In 1978 and 1981, a group of American scientists prepared Ascopyrone P by pyrolysis of amylopectin, amylose and cellulose at the Wood Chemistry laboratory in Montana, with the intention of using Ascopyrone P as a starting material for organic synthesis [Shafizadeh, F., Furneaux R.H., Stevenson, T.T., and Cochran, T.G., 1,5-Anhydro-4-deoxy-D-*glycero*-hex-1-en-3-ulose and other pyrolysis products of cellulose, Carbohydr. Res. 67(1978): 433-447; Stevenson, T.T., Stenkmap, R.E., Jensen, L.H., Cochran, T.T., Shafizadeh, F., and Furneaux R.H., The crystal structure of 1,5-anhydro-4-deoxy-D-*glycero*-hex-1-en-3-ulose, Carbohydr. Res. 90(1981): 319-325]. They characterised Ascopyrone P by, for example, ^{1}H and ^{13}C NMR, and IR spectroscopy techniques. A 3-dimensional structure of Ascopyrone P was provided. The yield of Ascopyrone P obtained by pyrolysis was under 3% and complicated separation methods had to be used.

The natural occurrence of Ascopyrone P in some species of very scarcely studied fungi collected from the Alps has been taught [M.-A. Baute, G. Deffieux, J. Vercauteren, R. Baute, and Badoc A., Enzymatic activity degrading 1,4-α-glucans to Ascopyrones P and T in *Pezizales* ad *Tuberales*, *Phytochemistry*, 33 (1993): 41-45]. The occurrence of Ascopyrone P in fungi immediately prompted the hypothesis that Ascopyrone P would act as an antibiotic. However, Ascopyrone P did not function satisfactorily as an antibiotic in the disclosed tests.

Many of the compounds of the present invention can be derived from 1,5-anhydrofructose. 1,5-Anhydrofructose is monoketo sugar found in bacteria, red algae, fungi and mammals. In red algae and fungi 1,5-anhydrofructose is produced by the action of α-1,4-glucan lyase [EC 4.2.2.13] from floridean starch and glycogen, respectively.

When the compound of the present invention is prepared from 1,5-anhydro-D-fructose, preferably the 1,5-anhydro-D-fructose is prepared in accordance with GB-A-2296717. In other words, preferably the 1,5-anhydro-D-fructose is prepared by a method comprising treating an α-1,4-glucan with the enzyme α-1,4-glucan lyase characterised in that enzyme is used in substantially pure form.

Ascopyrone P and Ascopyrone T can be produced enzymatically from 1,5-anhydro-D-fructose using cell-free extract prepared from the fungi of the order *Pezizales*, such as *Plicaria leiocarpa* and *Anthracobia melaloma*, and the order of Tuberales, such as, *Tuber melanosporum*. Ascopyrone T_1 is the dihydrate form of Ascopyrone T, whereas Ascopyrone T_2 and T_3 are the tautomeric monohydrate forms of Ascopyrone T.

Ascopyrone M can be produced from 1,5-anhydro-D-fructose by EDTA-sensitive dehydratases isolated from the fungi Morels, such as *Morchella vulgaris, Gyromitres, pezizes*, such as *Peziza echinospora.*

Ascopyrone M, P and T can also be produced chemically by treating 1,5-anhydro-D-fructose with alkali under mild conditions [Studies on the degradation of some pentoses and of 1,5-anhydro-D-fructose, the product of the starch-degrading enzyme a-1,4-glucan lyase; Thesis, Ahmad, T., The Swedish University of Agricultural Sciences, Sweden, 1995].

When the compound of the present invention is prepared by chemical means, it may be prepared in accordance with one of the following methods:

(1) Ascopyrone P may be produced by treating 1,5-anhydro-D-fructose with non-aqueous

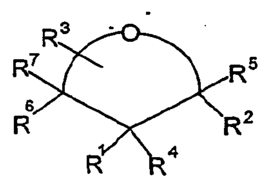
acid at elevated temperature, for example at 70 °C.

(2) Ascopyrones (for example, Ascopyrone P, T and M) may be produced from 1,5-anhydro-D-fructose by alkaline treatment according to Ahmad, T., 1995.

The structures of all ascopyrones produced were confirmed by NMR techniques.

Preferably, the compound of the present invention is prepared by enzymatic means as disclosed in M.-A. Baute *et al*, [*Phytochemistry*, 33 (1993): 41-45). For example ascopyrones (such as, Ascopyrone P, T and M) may be produced from 1,5-anhydro-D-fructose using enzymatic methods as disclosed in M.-A. Baute *et al.*

In another preferred aspect, the cyclic compound of the invention is of Formula IV,



IV

or a derivative thereof,

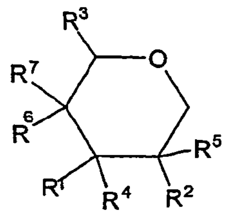
wherein R^1 and R^2 are independently selected from -OH, =O, and OR', wherein R' is H or -COR", and R" is C_{1-10} alkyl;

wherein R^3 is a substituent comprising an -OH group;

wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound;

wherein R^6 and R^7 are each independently selected from H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound.

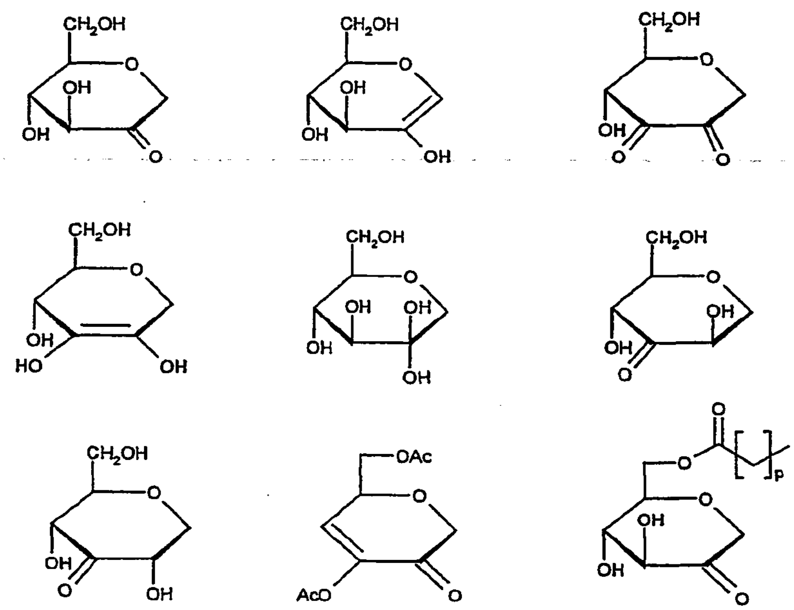
In a more preferred aspect, the cyclic compound of the invention is of formula V,



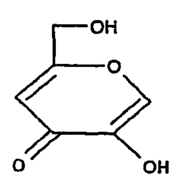
V

or a derivative thereof, wherein R^1, R^2, R^3, R^4, R^5, R^6 and R^7 are as defined above.

Even more preferably, the cyclic compound of the invention is selected from one or more of the following:



p = 1,2....24



In a particularly preferred aspect of the invention, R^3 is or comprises a CH_2OH group.

Preferably, the cyclic compound of the invention comprises a five or a six membered ring.

In a particularly preferred embodiment of the invention, the cyclic compound having Formula I has an antimicrobial effect against a micro-organism selected from *Listeria monocytogenes, Listeria innocua, Salmonella* Typhimurium, *Salmonella* sp., *Bacillus cereus, Bacillus subtilis, Saccharomyces cerevisiae, Saccharomyces cerevisiae* var. *paradoxus, Saccharomyces carlsbergensis Pseudomonas fluorescens, Clostridium sporogenes, Lactobacillus sake, Brochothrix thermosphacta, Micrococcus luteus, Yersinia enterocolitica, Enterobacter aerogenes, Zygosaccharomyces bailii.*

The cyclic compound of the invention may be used alone, or in combination with other components, for example, one or more chelators (such as EDTA sodium salt, polyphosphate or citrate) and/or one or more antioxidants (such as ascorbate, isoascorbate, ascorbate palmitate, BHA or BHT).

Tests indicate that APP is stable in water at 24 °C for 2 weeks, but completely disappears after 2 months. It has been reported that APP is stable at pH 1.5-5.5, and less stable at increasing pH. At pH 11-12.5 APP has a half-life of 5 h. Since most food is acidic or neutral, the stability may therefore be improved by using APP in combination with an antioxidant such as those listed hereinbefore.

In one preferred embodiment, the compound is used in combination with one or more preservatives. By way of definition, in the broadest sense, the term "preservative" is intended to encompass all substances which inhibit the development of, or kill, micro-organisms. In a narrower sense, it is generally understood that preservatives are used in concentrations of 0.5 % or less. Food additives which are allowed to be used as preservatives are listed in the Regulation No. 95/2/EG of the European Parliament and Council of 20 February 1995, relating to food additives other than colouring agents and sweeteners.

Typical food preservatives permitted in the EU which are suitable for use in combination with the compounds of the invention include sorbic acid, benzoic acid, PHB ester (p-hydroxybenzoate), and sulphur dioxide. The mode of action of these preservatives, together with their range of effects are listed below.

<u>Sorbic Acid</u> (E200 to 203):

Mode of action: inhibits different enzymes in the cells of the micro-organisms.

Range of effects: mainly against yeasts and moulds as well as catalase-positive bacteria. Catalase-negative bacteria as well as lactic acid bacteria and clostridia are not inhibited.

Effective concentration: 500 - 3000 ppm.

Permitted maximum quantities in food: up to 2000 ppm in potato dough, processed cheese, packed bread, fine bakery products, emulsified sauces etc.

<u>Benzoic Acid</u> (E210 to 213):

Mode of action: inhibits exchange of oxygen through the cellular membrane and affects the enzymatic structure.

Range of effects: for acid products only, up to approx. pH 4.5; inhibits yeasts and moulds, restricted inhibition of bacteria (no, or only very little, inhibition of lactic acid bacteria and clostridia).

Permitted maximum quantities in food: 500 ppm in aspic, fruit preparations, marmalades etc.

<u>PHB Ester</u> (p-hydroxybenzoate) (E214 to 219)

Mode of action: damages the bacterial membrane because of the surface activity, poisonous to protoplasm because of protein denaturation.

Range of effects: mainly inhibits yeasts and fungi, but also Gram-positive bacteria in a pH range between 3.0 and 8.0.

Effective concentration: sensorical influence at concentrations beyond approx. 0.08 %.

<u>Sulphur Dioxide</u> (E220 to 224; E 226 to 227)

Mode of action: depends on pH to a great extent, in practice it is only effective at acidic pH values (< 4,0). Very complex mechanisms.

Range of effects: mainly antibacterial, above all against Gram-negative, aerobic bacteria.

Effective concentrations: 250 - 500 ppm for inhibition of aerobic, Gram-negative bacteria, 800 - 2000 ppm against Gram-positive bacteria, yeasts, and moulds.

Permitted maximum quantity in food products: max. 2000 ppm in dry fruits, grape juice concentrate for home production of wine, in some cases only max. quantities of 20 - 30 ppm are permitted.

For more specific applications, the compounds of the present invention may also be used in combination with the following preservatives: biphenyl, diphenyl, orthophenylphenol, thiabendazol, nisin, natamycin, hexamethylentetramine, dimethyldicarbonate, boric acid, sodiumtetraborate, nitrite, propionic acid and propionate, and lysozyme. The mode of action of these preservatives, together with their range of effects and specific uses are listed below.

Biphenyl, Diphenyl (E 230)
Range of effects: Inhibition of moulds.
Substance for treatment of fruits: surface treatment of citrus fruits.
Permitted maximum quantity: 70 ppm

Orthophenylphenol (E 231 / E 232)
As with E230, limited to treatment of fruits as a surface treatment for citrus fruits.

Thiabendazol (E 233)
Surface treatment of citrus fruits and bananas.

Nisin (E 234)
Mode of action: Disturbance of membrane functions.
Range of effects: Gram-positive bacteria, no influence on Gram-negative bacteria.
Permitted maximum quantity in food products (EU): 3ppm in semolina pudding and similar products, 12.5 ppm (= 12.5 IU/g) in ripened cheese and processed cheese, 10 ppm in clotted cream, 10 ppm in mascarpone.

Natamycin (Pimaricin) (E235)
Mode of action: specifically attacks cell membrane, where - in general - an interaction with sterines occurs which increases the permeability of the membrane.

Range of effects: Moulds and yeasts, not effective against bacteria. Usual dosage rates are below approx. 50 mg / l. Maximum level is 1 mg/dm^2 on the surface, with a maximum penetration of 5 mm.

Applications: surface treatment of hard, semi-hard and semi-soft cheese and of dried, cured sausages.

Hexamethylentetramine (E 239)

Hexamethylentetramine is formed by adding ammonia to formaldehyde in an aqueous solution. The microbicidal effect is due to the formaldehyde.

Permitted only for Provolone cheese (25 ppm residual quantity).

Dimethyldicarbonate (E 242)

Permitted only for non-alcoholic drinks, non-alcoholic wine, and liquid concentrate.

Boric Acid, Sodiumtetraborate (E284 / E 285)

Permitted only for caviar.

Nitrite (E 249 and E 250)

Permitted in the form of nitrite curing salt for treatment of meat products ("red products"). For cured and dried meat products which are not heat treated and for other cured meat products an addition of 150 ppm has been fixed as a guideline. These concentrations do not show a preservative effect. They are mainly added for their technological properties (formation of colour, taste) as well as for their antioxidant effects.

Propionic Acid and Propionate (E 280, E 281, E 282, and E 283)

Mode of action: similar to sorbic acid, pH < 4.5 is optimal.

Accumulation in the cell leads to inhibition of enzymes.

Range of inhibition: moulds are inhibited at an pH of 5.5 by concentrations of 125 to 12500 ppm, for inhibition of bacteria higher concentrations are necessary (> 16000 ppm).

Application: Sliced and packaged bread.

Permitted maximum quantity: 3000 ppm.

Lysozyme (E 1105)

Permitted only for ripened cheese.

Permitted maximum quantity: quantum satis.

Studies by the applicant of the inhibitive effects of the present compounds have been tested in a medium (Elliker broth) with an almost neutral pH (pH 6.8) and have been shown to be effective against both Gram-positive and Gram-negative bacteria. As many of the preservatives described above show an inhibitory effect mainly at low pH, the use of the compounds of the present invention clearly broadens the potential range of applications.

In principle, the use of substances for chemical preservation depends on the following factors:

(a) Toxicological harmlessness

- the effects of the substance when applied acutely, subchronically, and for a long term period.
- Testing of acute toxicity (LD_{50}), cinetics and metabolism, pharmacological effects, genotoxicity, etc.

(b) Technological / food chemical aspects:

- Solubility in water: as growth takes place in the aqueous phase, a preservative has to be water-soluble
- Reaction with food ingredients, problem of off-flavours (sensory acceptance)
- Interferences with food ingredients (e.g. destruction of vitamin B1 by sulphuric acid)

The antimicrobial effectiveness of chemical substances in food and feed products is thus determined by a range of different factors. Among others, the composition of the population of micro-organisms, the composition of the food product (ingredients, pH, water activity, content of salt, etc.), the packaging, time-temperature-conditions, etc. are

key factors that influence the inhibitory activities of the antimicrobial agent.

The invention will now be described only by way of example, and with reference to the accompanying figures, wherein:

Figure 1	shows the antimicrobial activity of Ascopyrone P (APP) against *Salmonella* Typhimurium *DSMZ 554* (10e3 cfu/ml).
Figure 2	shows the antimicrobial activity of Ascopyrone P (APP) against *Salmonella* Typhimurium *DSMZ 554* (10e5 cfu/ml).
Figure 3	shows the effect of APP against *S.* Typhimurium S29 at 30 °C over a period of 24 h.
Figure 4	shows the effect of APP on the growth of *Br. thermosphacta* CRA7883 over a 72h period.
Figure 5	shows the effect of APP on the growth of *L. monocytogenes* 272 at 30 °C over a period of 24 h.
Figure 6	shows the effect of APP on the growth of *B. cereus* 204 at 30 °C over a period of 24 h.
Figure 7	shows the effect of APP on the growth of *Ps. fluorescens* 3756 at 30 °C over a period of 24 h.
Figure 8	shows the effect of APP on *L. monocytogenes* 272 in chicken soup at 8 °C (detection limit 10^2 cfu/g) over a 60 day period.
Figure 9	shows the effect of APP against *Ps. fluorescens* 3756 in chicken soup at 8°C (detection limit 10^2 cfu/g) over a 49 day period.
Figure 10	shows the effect of APP against Salmonella Typhimurium S29 in chicken soup at 8 °C. Detection limit 102 cfu/ml. Trial finished at 49 d
Figure 11	shows the effect of APP against B. cereus 204 spores in chicken soup at 8 °C. Detection limit 102 cfu/ml. Trial finished at 49 d
Figure 12	shows the effect of APP on *S.* Typhimurium S29 in a cooked mince beef slurry system at 8°C.
Figure 13	shows the effect of APP against Salmonella Typhimurium S29 in minced chicken slurry at 8 °C.

EXAMPLES

1. PRELIMINARY INVESTIGATION

Inhibition of Gram-positive and Gram-negative bacteria

Antimicrobial activity was investigated using *Salmonella* Typhimurium and *Listeria innocua*.

Bacterial growth was carried at 37°C for 24 hour for *S.* Typhimurium and 20 hours for *Listeria innocua*, both at an inoculation size of 10e3 or 10e5 cfu/ml (10^3 or 10^5 colony forming units per ml).

In both cases, APP showed a satisfactory inhibitory effect.

S. Typhimurium

For *S.* Typhimurium at 10e3 and 10e5, complete inhibition was observed at 2000 μg/ml and 4000 μg/ml (see Figures 1 and 2). Intermediate inhibition was observed at 500 μg/ml, with strong inhibition observed at 1000 μg/ml.

L. innocua

For *L. innocua* at 10e3, almost complete inhibition was observed at 2000 μg/ml, intermediate inhibition at 1000 μg/ml, and slight inhibition at 500 μg/mL At 10e5, intermediate inhibition was seen at 2000 μg/ml, slight inhibition at 1000 μg/ml, and almost no inhibition at 500 μg/mL

The tests described above were all performed under the optimal growth conditions for the bacteria at high inoculation levels. In food, the growth conditions for these bacteria will be less favourable and the inoculation level will be lower, therefore lower dosages will be needed to inhibit their growth. In other words, lower concentrations of the compounds of the invention will be necessary in food products, for a sufficient inhibition of food poisoning or food spoiling micro-organisms.

The above results clearly show that APP inhibits both Gram-negative bacteria of the *Enterobacteriaceae* type (*Salmonella* Typhimurium) and Gram-positive bacteria (*Listeria innocua*).

Further trials were undertaken to investigate the antimicrobial efficacy of ascopyrone P (APP) in laboratory media and in a range of food systems.

2. MATERIALS AND METHODS

2.1 TEST STRAINS

All micro-organisms were taken from storage at -80 °C. Most organisms were tested as vegetative cell suspensions from overnight broth culture. *Bacillus* and *Clostridium* species were tested as endospore suspensions prepared earlier and stored at 4 °C.

For broth cultures and Bioscreen testing most bacteria were grown in Brain Heart Infusion (BHI, Oxoid, pH 7.4). *Lactobacillus sake* A10 was grown in de Man, Rogosa, Sharpe medium (MRS, Oxoid). Yeasts were grown in Sabouraud Liquid medium (SLM, Oxoid). Most bacteria were cultured at 30 °C. Lactic acid bacteria were grown on solid medium in enriched CO_2 atmosphere. *Clostridium* species were grown in Reinforced Clostridial Medium (RCM) at 37 °C anaerobically. *Brochothrix thermosphacta,* fungi and yeasts were grown at 25 °C. Fungi were cultured on Malt Extract Agar (MEA, Oxoid).

2.2 ASCOPYRONE P (APP) SAMPLES

Sample E002012 was a dry powder that was dissolved in sterile de-ionised water. It was tested without filtration and used in the mini well diffusion test, initial Bioscreen runs (BS1211200; BS131200), and mini cidal experiment. The concentration of this sample was 49.3 mg/ml. Further APP samples were prepared as follows: 3.84 g of batch number APP20010213, as 5 x 4 ml volumes, was made up to a concentration of 169 mg APP/ml, and 5.5 g of batch number APP20010215, as 4 x 10 ml volumes, was made up to a concentration of 138 mg APP/ml. All samples were kept at -20 °C until use. Stock

solutions were made up in de-ionised water and filter sterilised. These samples were used in Bioscreen run BS010411 and BS010420. APP20010213 was used in the following trials: chicken soup at 8 °C (TCS010412; TCS 010430); raw meat (TRM010521). APP20010215 was used in Bioscreen run BS010510, *Clostridium* sensitivity tests and in the following trials: chicken soup at 8 °C (TCS010521); apple juice at 25 °C (TAJ010530); milk (TMBG010605), sausage meat slurry test (TSM010605); further raw meat tests (TRM010620) and cooked beef and chicken slurry tests (TCM010619; TCC010704).

2.3 *IN VITRO* GROWTH INHIBITION TEST METHODS TESTING

2.3.1 Well diffusion testing

Seeded 10 ml agar plates of various test organisms were prepared, with inoculation of either 20 µl spore suspension or overnight broth. This gave an inoculum level of ca. 10^5 - 10^6 cfu/ml. After the plates had set, small wells were cut, and 20 µl of APP sample was loaded. Plates were incubated overnight at appropriate temperature and examined after 1-2 days (after which microbial growth is clearly visible) for zones of inhibition.

2.3.2 Bioscreen testing

An automated Microbiology Reader Bioscreen C was used to measure growth curves of the strains in the presence and absence of APP. The Bioscreen C measures the development of turbidity (i.e. growth) kinetically by vertical photometry in 200 wells of a honeycomb microtitre plate, simultaneously. The system consists of a Bioscreen C analyser, which is an incubator and measurement unit, integrated with a PC, software (BioLink v 5.30), printer and a 'Honeycomb 2' cuvette multiwell plate. Growth curve data can be analysed within the BioLink software or exported to programs such as Excel.

Broth culture media were dispensed in 270 µl volumes into the wells as directed. Serial dilutions of a filter-sterilised APP stock solution were then dispensed into the same wells, as appropriate. The wells were inoculated with 30 µl of an appropriately diluted overnight broth culture or spore suspension, to give a final inoculum level of ca. 10^3 cfu/ml. The tests were incubated in the Bioscreen C for either 24 h at 30 °C, or 72 h at 25 °C

(depending on the micro-organism under investigation) with readings taken every 20 minutes after the trays were shaken. After the incubation period was complete the data were exported to Excel for analysis.

2.3.3. Sensitivity testing for *Clostridium* species

Stock solutions of APP215 (0.5, 1 and 2%) were prepared and filter-sterilised. Cooked meat medium (CMM, Oxoid) was prepared by distributing 1 g of the medium to individual test tubes, which were then filled with 8.9 ml water. After autoclaving 100 µl of the APP stock solutions were added, to give final concentrations of 0, 500, 1000 and 2000 ppm APP. The tests were inoculated with 100 µl of *Clostridium* spores, to give an inoculum level of ca. 10^3 cfu/ml. The tubes were overlaid with 3 ml of 2% agar to maintain anaerobicity. The tests were incubated at 37 °C, and examined daily for signs of growth (turbidity, gas production).

2.4. *IN VITRO* CIDAL EXPERIMENT

100 µl of SDW (sterile deionised water) or APP sample E002012 was added to 890 µl 10 mM HEPES buffer (pH 7). 10 µl of an overnight culture of *L. monocytogenes* S23 was added to the test, which was incubated at ambient temperature for 2 h. After this time the bacteria were enumerated by viable count. The concentration of the APP sample was 24.7 µg/ml.

2.5 *IN VIVO* (FOOD) GROWTH INHIBITION TESTS

2.5.1 Food testing in chicken soup

A local supermarket brand of cream of chicken soup was used as a model food system. 600 g packs were purchased from the local supermarket. This was a 'fresh home-made type' of soup that was stored in the chill cabinet, with a short shelf life. It was chosen because it is a rich, nutritious food system containing a range of food components including meat, dairy and vegetable.

Ingredients were labelled as: chicken stock (water, duck fat, chicken, salt, flavourings, yeast extracts, pork gelatine, glucose syrup, vegetable concentrate, milk protein, sugar, malto-dextrin, vegetable oil, citric acid, lactose, chicken fat), milk, chicken (9%), cream (8%), onion, potato, vegetable oil, modified maize starch, wheat flour, lemon juice, salt, white pepper. pH 5.912. Protein: 3.3%; carbohydrate: 4.7%; fat: 7.2%; fibre: 0.2%; sodium: 0.1%

For the test the soup was mixed 2:1 in water (to facilitate sampling and mixing) and sterilised by autoclaving at 121 °C for 15 minutes. For each experiment the pH of the soup was recorded; this was between pH 5.79-5.88.

For TCS010412/8 (which was incubated at 8 °C), APP batch number APP20010213 (concentration 169 mg APP/ml) was used to prepare tests containing 0, 2000 or 4000 ppm APP. 5 g of filter-sterilised APP stock solution was added to 94 g of soup, mixed and then 1 ml of a diluted inoculum suspension (cells or spores as appropriate) was added to give a test inoculum level of 10^3 cfu/g. The soup mixture was shaken vigorously to ensure homogeneous distribution of the APP and micro-organisms. The tests were tested by viable count and then incubated at 8 °C and tested on a twice-weekly basis.

For TCS010430/20 (incubated at 20 °C) samples were prepared in 18.8 g quantities, to which 1 g of a suitable filter-sterilised APP stock solution was added and an appropriately diluted 0.2 ml inoculum. Great care was again taken to ensure homogeneous distribution of the APP and micro-organisms. This test was sampled by viable count test on a daily basis.

For TCS010521/8 (incubated at 8 °C), samples were prepared in 27.8 g quantities. 1.5 g of an APP stock solution was added, and the tests inoculated with 0.3 ml of a suitably diluted cell or spore suspension to give a final inoculum of ca. 10^3 cfu/g. APP was tested at 0, 500 and 1000 ppm, and batch number APP20010213 (at 169 mg/ml) was used. The tests were sampled by viable count and then incubated at 8 °C and tested on a twice-weekly basis.

2.5.2 Test in apple juice [TAJ010530/25]

8.9 ml apple juice samples (pH 3.42) were prepared and autoclaved. Batch number APP20010215 (at 138 mg/ml) was used to prepare filter-sterilised stock solutions for 1 ml addition to the juice to achieve 0, 1000, 2000 ppm APP. Tests were inoculated with 100 µl of an appropriately diluted overnight SLM broth of yeast culture or ascospore suspension of *Byssochlamys* spores prepared earlier. The tests were incubated at 25 °C, and examined daily for signs of growth (e.g. turbidity, mycelium development).

2.5.3 Test in milk [TMGB010605]

8.9 ml samples of UHT milk containing 0.5% glucose and the indicator bromocresol purple (0.01%) were prepared and sterilised by autoclaving. The pH before APP addition was pH 6.65. Appropriate stock solutions of APP batch number APP20010215 (at 138 mg/ml) were prepared and filter sterilised. 1 ml of each was added to the milk to prepare tests containing 0, 1000 and 2000 ppm APP. Tests were then inoculated with 100 µl of diluted overnight broth cultures or spore suspensions to give a final inoculum level of ca. 10^3 cfu/ml. These and uninoculated tests were incubated at 20 and 8 °C and examined daily for visible changes compared to uninoculated control.

2.5.4. Test in cooked sausage meat slurry [TSM010605]

500 g of bologna sausage meat mixture was mixed with 500 g Brain Heart Infusion broth (BHIB) to make a homogeneous slurry. The bologna sausage meat mix comprised (g per kg): lean beef 320; lean pork 202; lard 250; water 40; glucose 5; starch 155; spice mix 3.75; sodium glutamate 0.5; sodium erythorbate 0.545; sodium di-phosphate 3; NaCl 20; sodium nitrite 0.15. 200 g samples of the meat slurry were autoclaved. Whilst the meat was still warm it was re-mixed thoroughly. When the meat had cooled it was inoculated with 2 ml of 10^5 cfu/ml spore or cell suspension, and re-mixed again. Each inoculated meat sample was divided into 3 x 50 g samples, and 1 ml of either SDW, 2.5% or 5% APP solution (batch number APP 20010215) was added to prepare meat samples containing 0, 500 and 1000 ppm APP. The meat slurry was again shaken thoroughly to ensure even mixing, an initial viable count was taken and the samples incubated at 8 °C. Samples were taken at twice-weekly intervals. The pH of the sausage meat slurry was 6.2.

2.5.5. Test in cooked minced beef slurry [TCM010619]

1 kg of minced beef, purchased from the local butcher, was mixed with 1 litre of BHI. 200 g portion of this meat slurry was autoclaved. When the meat had cooled, the pH was recorded (pH 6.24), and each pot was inoculated with 2.0 ml of 10^5 spores or cells and the meat re-mixed thoroughly. Each inoculated 200 g pot was distributed as 3 x 50 g portions. To each pot 1 ml of SDW, 5% or 10% APP (Batch No. APP20010215) was added, and the meat was again mixed thoroughly. An initial viable count was performed, the meat was incubated at 8 °C and sampled twice weekly. The pH of the cooked minced beef slurry was 6.24.

2.5.6. Cooked minced chicken slurry [TCC010704]

1 kg of minced chicken (mainly lean meat) was purchased from the local butcher. This was mixed with 1 litre of BHI, mixed and dispensed as 100 g samples prior to autoclaving. When still warm, the meat was re-mixed thoroughly. When cooked, the meat was inoculated with 1 ml of 10^5 spores or cells (from an overnight broth), and re-mixed. The samples were then divided into 3 x 25 g portions, to which 0.5 ml of filter-sterilised APP20010215 was added (as 5% or 10% stock solutions) or water. An initial viable count was taken, and the samples then incubated at 8 °C and tested twice weekly. The pH of the chicken slurry was 6.42.

2.5.7. Uninoculated test in raw meat [TRM010521]

300 g of raw minced beef was purchased from the local butcher. 10 g of either a 2% (20,000 ppm) stock solution of APP2001213 or water was added, and the meat mixed thoroughly. Initial counts were carried out, and then the meat was incubated at 8 °C and sampled for 3 consecutive days. The pH of the raw meat was 5.3.

2.5.8. Inoculated test in raw meat test [TRM010620]

Fresh beef steak was purchased from the local butcher, and minced on clean machinery. The raw meat (ca. pH 6.2) was divided into 8 x 90 samples and inoculated–with 10^3 cfu/ml of test organisms. The meat was mixed thoroughly using a stomacher. 10 g of SDW or 2% APP (Batch number APP20010215) was added, and the meat was again

thoroughly mixed. An initial viable count was taken, the meat was incubated at 8 °C, and sampled on the following days using non-selective agar (MPCA) and selective agar: XLD for *Salmonella*; Oxford selective agar (*Listeria monocytogenes*); and STAA agar (*Brochothrix thermosphacta*). The pH of the raw meat was pH 5.3.

3. RESULTS

3.1. *IN VITRO* GROWTH INHIBITION RESULTS

3.1.1. Well diffusion results

The concentration of the test solution (unknown at time of testing) was 49.3 mg/ml (4.9%). All strains tested were sensitive to this level. They included: *Bacillus cereus* 204; *Clostridium sporogenes* Campden; *Listeria monocytogenes* S23; *Micrococcus luteus; Lactobacillus sake* A10; *Brochothrix thermosphacta* CRA7883; *Pseudomonas fluorescens* 327; *Saccharomyces carlsbergensis* CRA6413; *Saccharomyces cerevisiae* ATCC 9763.

3.1.2. Bioscreen test results: *in vitro* sensitivity of bacteria and yeasts

The Bioscreen test results are summarised in Tables 1-4 and Figures 3-7. Bioscreen run BS010510 was interrupted by power failure due to electrical storm overnight.

3.1.3. Sensitivity of *Clostridium* species to APP

No growth inhibition was observed at the highest level tested (2000 ppm APP20010215) against four strains of *Cl. sporogenes* (strains 1.221; Campden; ABC20; 4.440) and one strain of *Cl. tyrobutyricum* 2753 grown in cooked meat medium anaerobically at 37 °C.

3.2. *IN VITRO* CIDAL TEST RESULTS

After 2 h incubation at ambient temperature in buffer containing 24.7 µg/ml APP, the *Listeria* counts remained steady, (Table 5).

3.3. *IN VIVO* (FOOD) GROWTH INHIBITION TEST RESULTS

3.3.1. Results of chicken soup trials

The results of the chicken soup experiments incubated at 8 °C [TCS010412, TCS010521] and 20 °C [TCS010430] are shown in Tables 6-8, and Figures 8-11. The APP had no effect on the colour of the product.

3.3.2 Results of trials in apple juice incubated at ambient temperature [TAJ010530]

1000 and 2000 ppm APP controlled the growth of *Zygosaccharomyces bailii* CRA229 for 3-4 days. 2000 ppm controlled the growth of *S. cerevisiae* ATCC9763 for 1 day. No growth inhibition was achieved by 1000 or 2000 ppm APP against *Candida parapsilosis* 458; *Hanseniaspora uvarum* CBS5074; *Rhodotorula mucilaginosa* var *mucilaginosa* CBS8161; and the heat-resistant fungus *Byssochlamys fulva* 040021, and *B. nivea* 163642.

3.3.3 Results of milk trials incubated at 8 and 20°C [TMGB010605]

At 20 °C results were as follows:

No control observed at 2000 ppm for *L. monocytogenes, Br. thermosphacta, Lb. sake.*

Control for 2- 3 d at 2000 ppm for *Ps. fluorescens*

Control for 4 d at 1000 ppm, and 6 d at 2000 ppm for *B. cereus*

At 8 °C results were as follows:

No control observed at 2000 ppm for *L. monocytogenes, Lb. sake, B. cereus*

Control for 1 d at 2000 ppm for *Br. thermosphacta*

Control for > 7 d at 2000 ppm for *Ps. fluorescens*

3.3.4. Results of trial in cooked sausage meat slurry at 8 °C [TSM010605] (pH 6.2)

Results are summarised in Table 9.

3.3.5. Results of trial in cooked minced beef slurry [TCM010619]

Graphs of growth curves are shown in Figure 12 and summarised in Table 10.

3.3.6. Results of trial in cooked minced chicken slurry at 8 °C [TCC010704]

Growth curves are shown in Figure 13, and summarised in Table 11.

3.3.7. Results of uninoculated trial in raw meat at 8 °C [TRM010521] (pH 5.31)

Results are shown in Table 12: counts were identical in the control and APP samples. No negative effect of APP on the meat colour was observed.

3.3.8 Results of inoculated trial in raw meat at 8 °C [TRM010620]

Despite every precaution the TAVC of the meat was high; 10^6 cfu/g rising to > 10^7 cfu/g by the end of the trial. No significant effect of APP was observed. No negative effect was observed on the meat colour.

4.0 CONCLUSIONS

The results of the above tests are summarised in Table 13. The tests indicate that 2000 ppm APP is efficacious against the following micro-organisms: *B. cereus, L. monocytogenes, Ps. fluorescens, Salmonella* Typhimurium, *Lb sake, Y. enterocolitica, Br. thermosphacta, Z. bailii* and *S. cerevisiae.*

The observed inhibitory activity is probably bacteriostatic, but in the presence of additional preservative hurdles (particularly low temperature), a cidal effect is possibly achieved with time. Against some bacteria, efficacy of APP was better in a food system compared to in laboratory media, probably because the organism grew less well in the food. The efficacy of APP may vary with different foods due to the differences in their chemical compositions. For example, APP was less efficient in a sausage meat model than in chicken soup.

5.0 Tabulated Data

Table 1: Bioscreen results: effective levels of APP against Gram-positive bacteria

GRAM POSITIVE [Bacillus tested as spores]	APP LEVEL (ppm) CAUSING SIGNIFICANT OR TOTAL INHIBITION FOR TIME/TEMPERATURE INDICATED					
	BS010510 24 h at 30 °C. [APP20010213]		*BS010411* 24 h at 30 °C. [APP20010213]		*BS010411* 24 h at 30 °C. [APP20010215]	
	Total	Significant	Total	Significant	Total	Significant
B. cereus 204			> 4000	4000	4000	2000
B. cereus Campden			4000	2000	> 4000	2000
B. cereus 3.046	3000	2000				
B. subtilis Campden	3000	2000				
L. monocytogenes 358	3000					
L. monocytogenes 272			4000	2000	>4000	2000
L. monocytogenes S23			4000	2000	4000	1000
L. monocyt. Scott A	3000	2000				
L. monocyt. F6861	3000	2000				
Lb. sake A10			500	250	500	250

Table 2: Bioscreen results: effective levels of APP against *Br. thermosphacta*

	BS010420 72 h at 25 °C [APP20010213] APP (ppm)	
	Total	Significant
Br. thermosphacta CRA7883	4000	2000

Table 3: Bioscreen results: effective levels of APP against Gram-negative bacteria

GRAM NEGATIVES	APP LEVEL (ppm) CAUSING SIGNIFICANT OR TOTAL INHIBITION FOR TIME/TEMPERATURE INDICATED					
	APP20010213 BS010510 24 h/30		APP 20010213 BS010411 24 h/30 °C		APP 20010215 BS010411 24 h/30 °C	
	Total	Significant	Total	Significant	Total	Significant
Ps. fluorescens 3756			2000	1000	2000	1000
Ps. fluorescens 10460	2000	1000				
Ps. fluorescens 1331	1000					
Ps. fluorescens 327	1000					
Ent. aerogenes 10102	4000					
Y. enterocolitica S16	2000					

Salm. Typhimurium S29			4000	2000	> 4000	2000
Salmonella sp. S19	4000					

Table 4: Bioscreen results: effective levels of APP against yeasts

YEASTS	APP 213 BS010420 72 h/25 °C	
	Total	Significant
S. cerevisiae ATCC9763	> 4000	Enhanced growth
S. carlsbergensis CRA6413	> 4000	Enhanced growth
S cerevisiae H78	> 4000	4000
S cerevisiae var. *paradoxus* H103	> 4000	2000

Table 5: Results of cidal experiment with APP E002012

Strain	0 µg/ml APP	24.7 µg/ml APP
L. monocytogenes S23	4.3×10^6	1.3×10^7

Table 6: Chicken soup incubated at 8 °C. [TCS010412] APP20010213 tested at 0, 2000, 4000 ppm

Strain	Result
B. cereus 204	• Total inhibition by 2000 and 4000 ppm for > 60 days. • Counts remain at initial level , i.e. no spore kill.
L. monocytogenes 272	• Total inhibition by 4000 ppm for > 60 days. • Counts reach < 10^5 with 2000 ppm. • Counts undetectable after 20 days at 4000 ppm • Counts undetectable after ca 50 days at 2000 ppm
Ps. fluorescens 3756	• Total inhibition by 2000 and 4000 ppm for > 60 days. • Counts undetectable from day 1 in APP samples

Table 7: Chicken soup at 8 °C [TCSM010521]. APP215 tested at 0, 500, 1000 ppm

Strain	Result
B. cereus 204	• Total inhibition by 500 and 1000 ppm for > 27 days. • Counts remain at approx initial level , i.e. no spore kill.
L. monocytogenes 272	• No total inhibition by 1000 ppm, but slight reduction of growth rate • No effect with 500 ppm
L. monocytogenes S23	• No total inhibition by 1000 ppm • Slight reduction of growth rate with 500 and 1000 ppm

Ps. fluorescens 3756	• Total inhibition by 1000 ppm for > 27 days. • At 1000 ppm counts undetectable > 3 d • Little effect with 500 ppm
Ps. fluorescens 327	• Total inhibition by 1000 ppm for > 27 days. • At 1000 ppm counts undetectable > 7 d • Little effect with 500 ppm
Salmonella S19	• Total inhibition by 500 – 1000 ppm for 22 d • Slow growth in controls, reached 10^6 by 12 d
Salm Typhimurium S29	• Total inhibition by 1000 ppm for > 27 d • Total inhibition by 500 ppm for 25 d • Slow growth in controls, reached 10^6 by 14 d
S. cerevisiae 9763	• No effect with 500 and 1000 ppm

Table 8: Chicken soup incubated at 20 °C [TCSM010430]. APP20010213 tested at 0, 200, 500, 1000, 2000 ppm

Strain	Result
B. cereus 204	• Total inhibition by 2000 ppm for > 42 days. • Extension of lag with 1000 ppm • No significant effect with 500 ppm
L. monocytogenes 272	• No total inhibition but reduction of growth rate only by 2000 ppm • No significant effect by 1000 ppm
Ps. fluorescens 3756	• Total inhibition by 2000 ppm for > 42 days (counts undetectable). • No significant effect with 1000 ppm
Salmonella Typhimurium S29	• Reduction in final count by 2000 ppm (10^6 cf 10^9). After 8 d counts drop, undetectable by18 d. • No effect with 1000 ppm
S. cerevisiae 9763	• No effect with 2000 ppm

Table 9: Summarised results of cooked sausage meat slurry at 8 °C [TSM010605]

Strain	Result
B. cereus 204	• No total inhibition by 1000 ppm, 10^6 reached at 7 d • Controls reached 10^6 by 5 d.
L. monocytogenes 272	• No total inhibition by 1000 ppm, 10^6 reached at 7 d • Controls reached 10^6 by 7 d
Br. thermosphacta CRA78884	• No significant effect with 500 and 100 ppm
Lb. sake A10	• No effect with 500 and 1000 ppm
Salm Typhimurium S29	• Total inhibition by 500 for 25 d • Total inhibition by 1000 ppm for > 38 d • Slow growth in controls, reached 10^6 by 19 d

Table 10: Summary of results of trial in minced beef slurry at 8 °C [TCM010619]

Strain	Result
B. cereus 204	• 2000 ppm delayed growth to 10^6 by 7 d. • 1000 ppm extended lag phase.
L. monocytogenes 272	• No significant inhibition by 2000 ppm
Br. thermosphacta CRA78883	• No effect with 2000 ppm
Lb. sake A10	• No effect with 2000 ppm
Salm Typhimurium S29	• Total inhibition by 2000 ppm for > 44 d • Slow growth in controls, reached 10^6 by 13 d
Ps. fluorescens 3756	• Total inhibition by 2000 ppm for 27 d • 1000 ppm delayed growth by 17 d • Controls reached 10^6 by 15 d

Table 11: Summary of results of trial in minced chicken slurry at 8 °C [TCC010704]

Strain	Results after 13 d incubation
B. cereus 204	• Partial inhibition by 2000 ppm • Controls reached 10^6 by 5 d
L. monocytogenes 272	• Slight inhibition by 2000 ppm only
Salm Typhimurium S29	• Total inhibition by 1000 ppm for 25d • Total inhibition by 2000 ppm for >40 d • Controls not reached 10^6 by 14 d
Ps. fluorescens 327	• No total inhibition by 2000 ppm, 10^6 reached at 10 d • Controls reached 10^6 by 3 d

Table 12: Results of raw meat with added at 8°C. [TRM010521]

Date	Day	Viable counts (cfu/g)	
		0 ppm APP	2000 ppm APP
21/5	0	5.6×10^6	9.3×10^6
22/5	1	2.0×10^8	2.2×10^8
23/5	2	2.7×10^8	1.5×10^8
24/5	3	9.7×10^8	5.0×10^8

Table 13: Summary of test results for activity of 2000 ppm APP in food systems

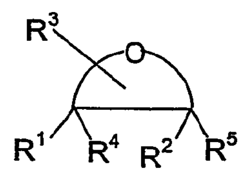
Food/test systems	Effect on growth of target organism by 2000 ppm APP		
	Total inhibition	Partial inhibition	No inhibition
Lab media at optimum temperature	*Lb. sake* *Ps. fluorescens* *Y. enterocolitica*	*B. cereus* *L. monocytogenes* *Br. thermosphacta* *Salmonella* spp	Most yeasts (enhanced growth) All *Clostridium* spp

Chicken soup at 8 °C pH 5.8	*B. cereus* *L. monocytogenes* *Ps. fluorescens* *Salmonella* spp		*S. cerevisiae* (1000 ppm)
Chicken soup at 20 °C pH 5.8	*B. cereus* *Ps. fluorescens*	*L. monocytogenes* *Salmonella*	*S. cerevisiae*
Apple juice at ambient pH 3.4	-	*Z. bailii* *S. cerevisiae*	*Candida parapsilosis* *H. uvarum* *Rh. mucilaginosa* *Byssochlamys fulva*
Milk at 20 °C pH 6.65	-	*Ps. fluorescens* *B. cereus*	*L. monocytogenes* *Br. thermosphacta* *Lb. sake*
Milk at 8 °C pH 6.65	*Ps. fluorescens*	*Br. thermosphacta*	*L. monocytogenes* *Lb. sake* *B. cereus*
Cooked sausage meat slurry at 8 °C pH 6.2	*S.* Typhimurium (1000)	*B. cereus* (1000) *L. monocytog.* (1000)	*Br. thermosphacta* (1000) *Lb. sake* (1000)
Cooked minced beef slurry at 8 °C pH 6.24	*S.* Typhimurium *Ps. fluorescens*	*B. cereus*	*Br. thermosphacta* *Lb. sake* (*L. monocytogenes*)
Cooked minced chicken slurry at 8 °C pH 6.42	*S.* Typhimurium	*Ps. fluorescens* *B. cereus*	(*L. monocytogenes*)
Raw uninoculated meat at 8 °C. pH 5.3	-	-	TAVC
Raw inoculated meat at 8 °C. pH 5.3	-		TAVC *L. monocytogenes* *Salmonella* Typhimurium *Br. thermosphacta*

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Modifications of the described modes for carrying out the invention which are obvious to those skilled in the relevant are, or related fields, are thus intended to fall within the scope of the following claims.

CLAIMS

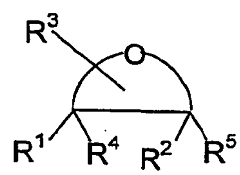
1. An antimicrobial composition for use against a micro-organism selected from *Listeria, Salmonella, Bacillus, Saccharomyces, Pseudomonas, Clostridium, Lactobacillus, Brochothrix, Micrococcus, Yersinia, Enterobacter* and *Zygosaccharomyces,* said composition comprising a cyclic compound having Formula I,



I

or a derivative thereof,
wherein R^1 and R^2 are independently selected from -OH, =O, and OR', wherein R' is H or -COR", and R" is C_{1-10} alkyl;
wherein R^3 is a substituent comprising an -OH group;
wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound.

2. A process for preventing and/or inhibiting the growth of, and/or killing, micro-organisms in a material, the process comprising the step of contacting the material with a cyclic compound having Formula I,

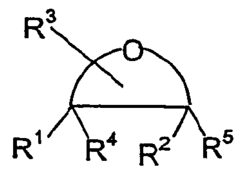


I

or a derivative thereof,
wherein R^1 and R^2 are independently selected from -OH, =O, and OR', wherein R' is H or -COR", and R" is C_{1-10} alkyl;
wherein R^3 is a substituent comprising an -OH group;
wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound;

wherein said micro-organism is selected from *Listeria, Salmonella, Bacillus, Saccharomyces Pseudomonas, Clostridium, Lactobacillus, Brochothrix, Micrococcus, Yersinia, Enterobacter* and *Zygosaccharomyces.*

3. Use of a compound having Formula I, or a derivative thereof,



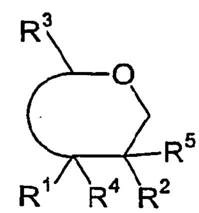
I

wherein R^1 and R^2 are independently selected from -OH, =O, and OR', wherein R' is H or -COR", and R" is C_{1-10} alkyl;
wherein R^3 is a substituent comprising an -OH group;
wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound;
for preventing and/or inhibiting the growth of, and/or killing, micro-organisms in a material, wherein said micro-organism is selected from *Listeria, Salmonella, Bacillus, Saccharomyces, Pseudomonas, Clostridium, Lactobacillus, Brochothrix, Micrococcus, Yersinia, Enterobacter* and *Zygosaccharomyces.*

4. The invention according to any one of the preceding claims wherein said material is a foodstuff or feed.

5. The invention of any one of the preceding claims wherein said cyclic compound is a compound having formula I, or a derivative thereof,
wherein R^1 and R^2 are independently selected from -OH, =O;
wherein R^3 is a substituent comprising an -OH group;
wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound.

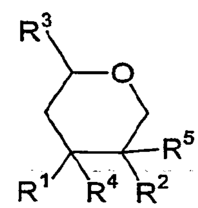
6. The invention of any one of the preceding claims wherein the cyclic compound is a compound having Formula II



II

or a derivative thereof, wherein R^1, R^2, R^3, R^4, and R^5 are as defined in the preceding claims.

7. The invention of any one of the preceding claims wherein the cyclic compound is a compound having Formula III

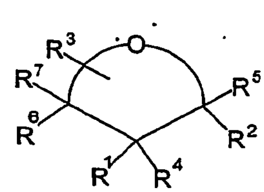


III

or a derivative thereof; wherein R^1, R^2, R^3, R^4, and R^5 are as defined in the preceding claims.

8. The invention of any one of the preceding claims wherein the compound is selected from Ascopyrone P, Ascopyrone M, Ascopyrone T, Ascopyrone T_1, Ascopyrone T_2, Ascopyrone T_3, and mixtures thereof.

9. The invention of any one of claims 1 to 3 wherein said cyclic compound is of Formula IV,



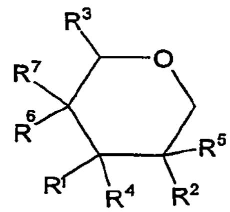
IV

or a derivative thereof,

wherein R^1 and R^2 are independently selected from -OH, =O, and OR', wherein R' is H or -COR", and R" is C_{1-10} alkyl;

wherein R^3 is a substituent comprising an -OH group;
wherein R^4 and R^5 are each independently selected from a hydrocarbyl group, H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound;
wherein R^6 and R^7 are each independently selected from H, OH or =O, or represent a bond with an adjacent atom on the ring of the cyclic compound.

10. The invention of any one of claims 1 to 9 wherein said cyclic compound is of formula V,



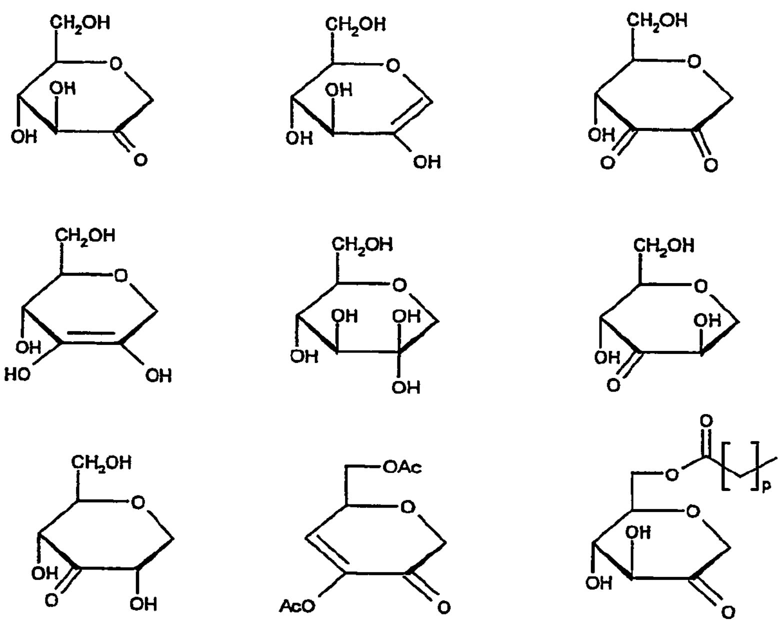
V

or a derivative thereof, wherein R^1, R^2, R^3, R^4, R^5, R^6 and R^7 are as defined in claim 9.

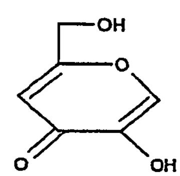
11. The invention according to any one of the preceding claims wherein the derivative of the compound of formula I is an ester.

12. The invention according to claim 11 wherein the ester is formed from an -OH substituent on the cyclic compound, and wherein said ester is of the formula $-(CH_2)_n-OC(O)-(CH_2)_pCH_3$, wherein n and p are each independently from 1 to 24.

13. The invention of any one of claims 9 to 12 wherein said cyclic compound is selected from one or more of the following:



p = 1,2....24



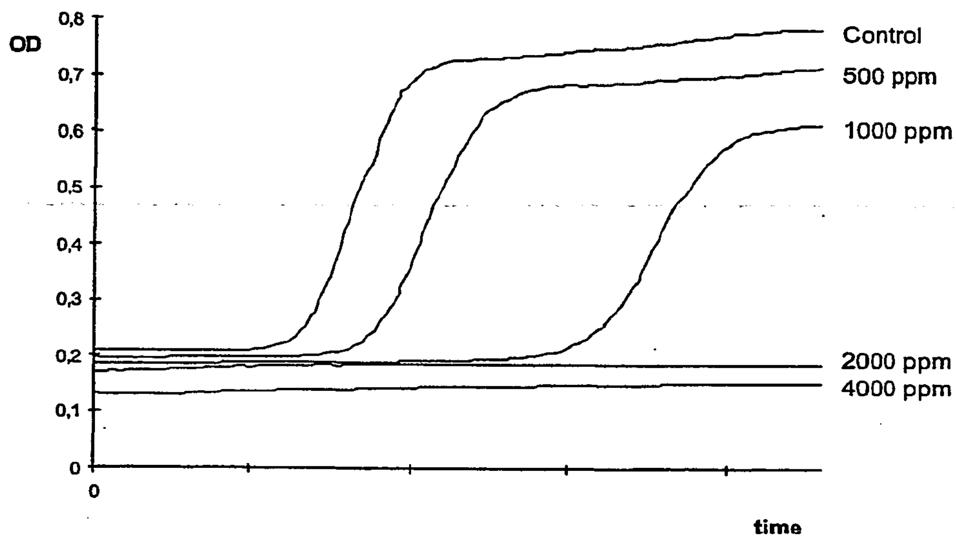
14. The invention of any one of the preceding claims wherein R^3 is or comprises a CH_2OH group.

15. The invention of any one of the preceding claims wherein the cyclic compound comprises a five or a six membered ring.

16. The invention according to any preceding claim wherein the cyclic compound having formula I has an antimicrobial effect against a micro-organism selected from *Listeria monocytogenes, Listeria innocua, Salmonella* Typhimurium, *Salmonella* sp., *Bacillus cereus, Bacillus subtilis, Saccharomyces cerevisiae, Saccharomyces cerevisiae* var. *paradoxus, Saccharomyces carlsbergensis, Pseudomonas fluorescens, Clostridium sporogenes, Lactobacillus sake, Brochothrix thermosphacta, Micrococcus luteus, Yersinia enterocolitica, Enterobacter aerogenes* and *Zygosaccharomyces bailii.*

17. The invention according to any preceding claim wherein said compound of formula I is used in combination with one or more of an antioxidant, a preservative and/or a chelator.

FIGURE 1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0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
0
time
Control
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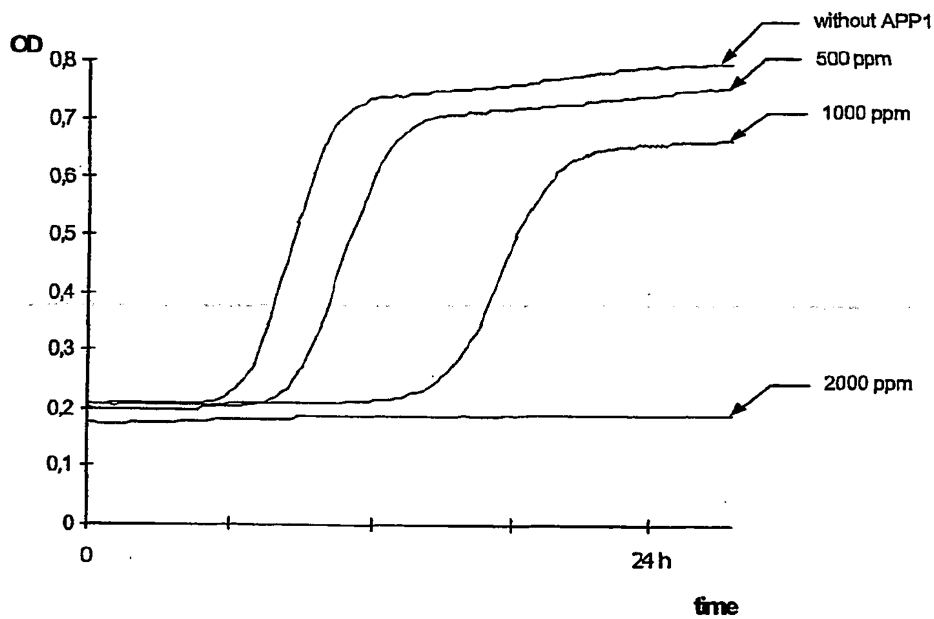
1000 ppm
2000 ppm
4000 ppm

FIGURE 2


OD
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0,7
0,6
0,5
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0,1
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0
24 h
time
without APP1
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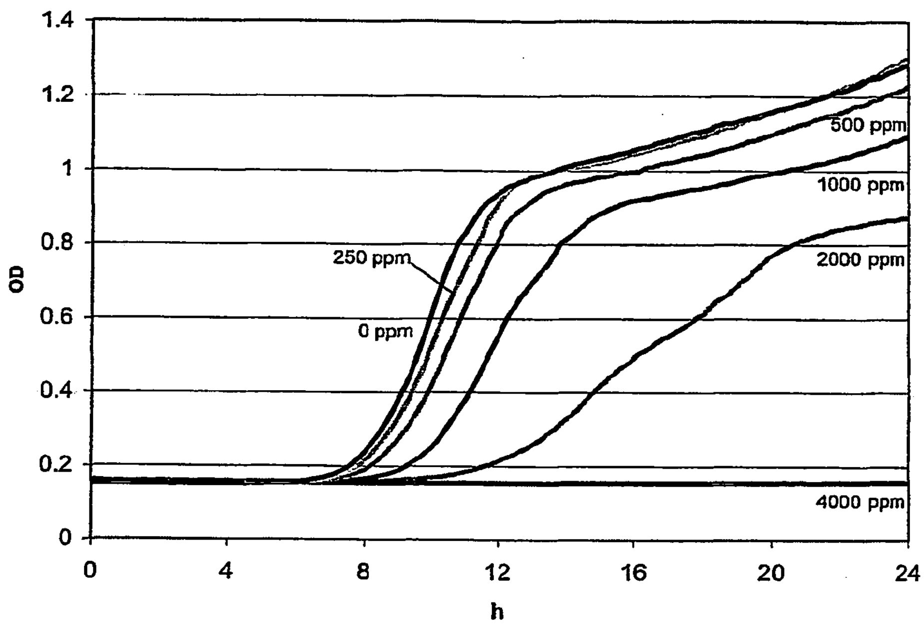
1000 ppm
2000 ppm

FIGURE 3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0.2
0
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4
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12
16
20
24
h
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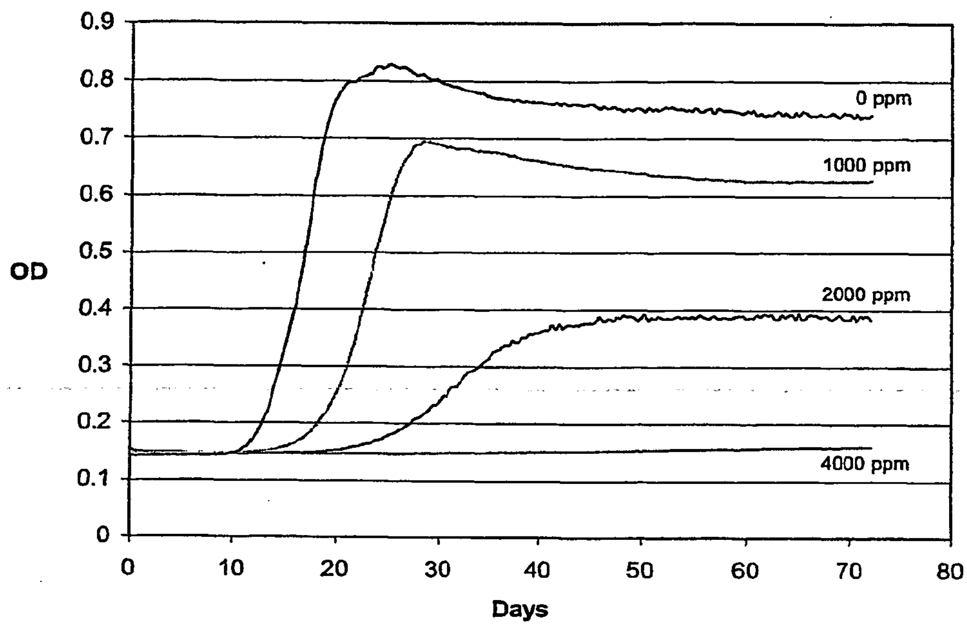
0 ppm
500 ppm
1000 ppm
2000 ppm
4000 ppm

FIGURE 4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0.8
0.7
0.6
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0.3
0.2
0.1
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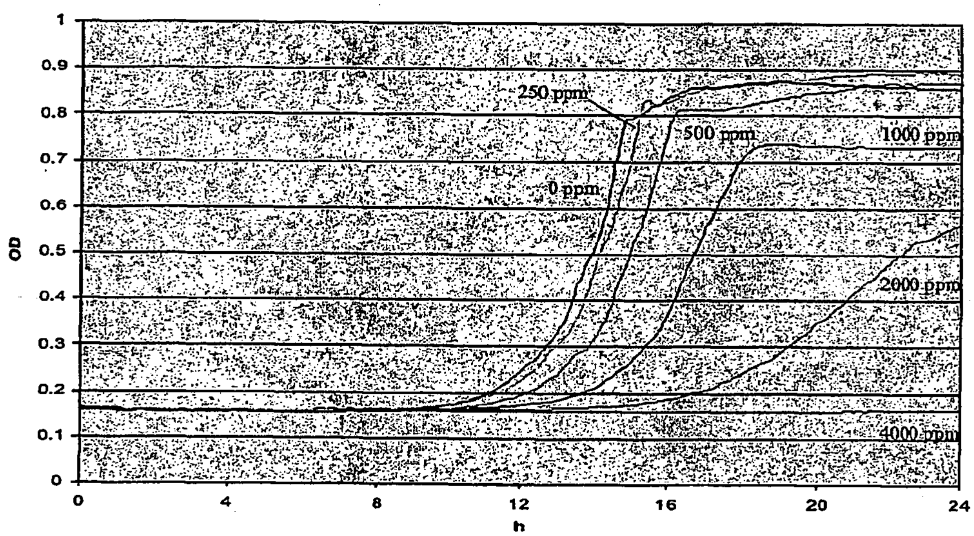
2000 ppm
4000 ppm
0
10
20
30
40
50
60
70
80
Days

FIGURE 5


250 ppm
500 ppm
1000 ppm
0 ppm
2000 ppm
4000 ppm
OD
h
0
0.1
0.2
0.3
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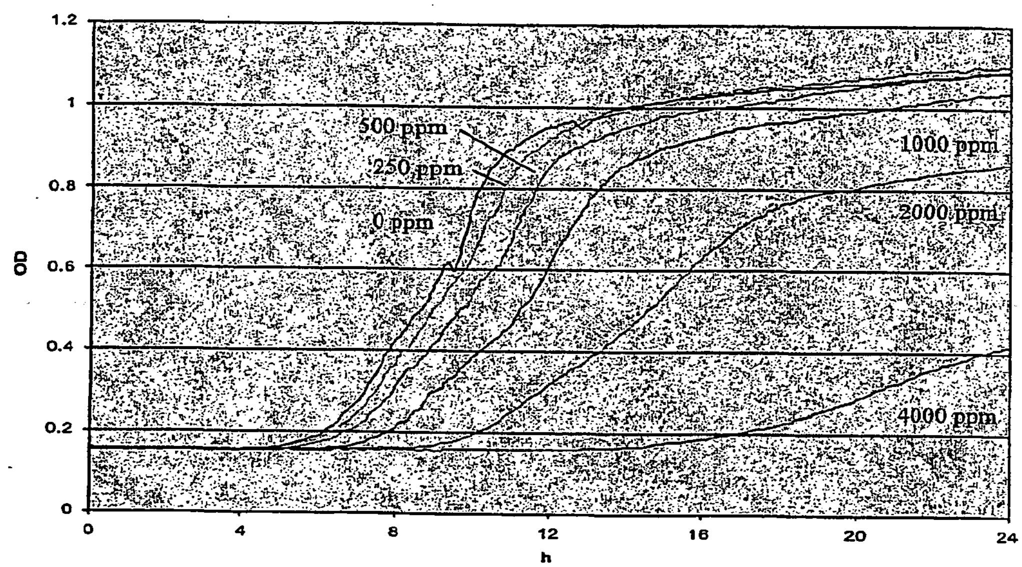
0.6
0.7
0.8
0.9
1
0
4
8
12
16
20
24

FIGURE 6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4
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12
16
20
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h
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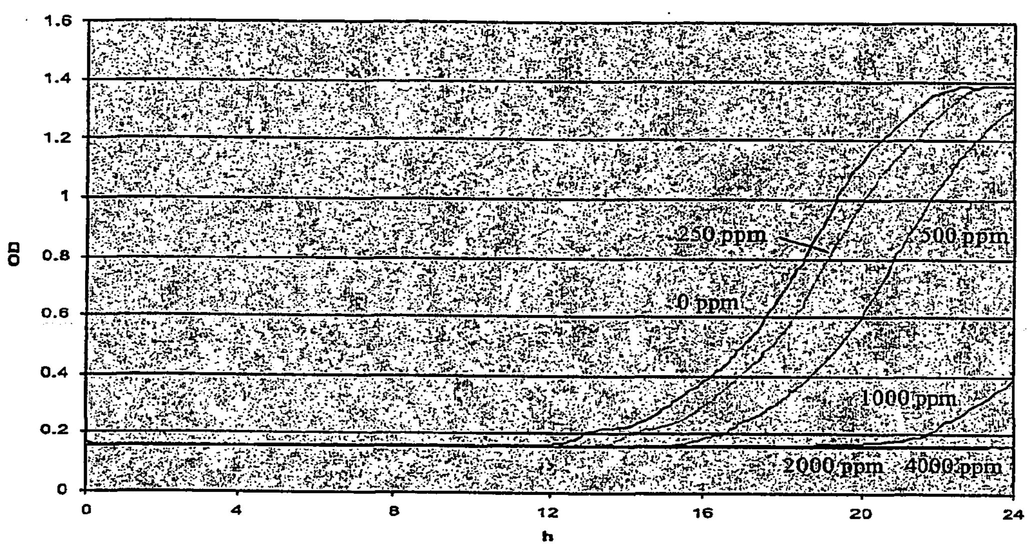
250 ppm
0 ppm
1000 ppm
2000 ppm
4000 ppm

FIGURE 7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1.2
1
0.8
0.6
0.4
0.2
0
OD
0
4
8
12
16
20
24
h
250 ppm
500 ppm
0 ppm
1000 ppm
2000 ppm
4000 ppm

FIGURE 8

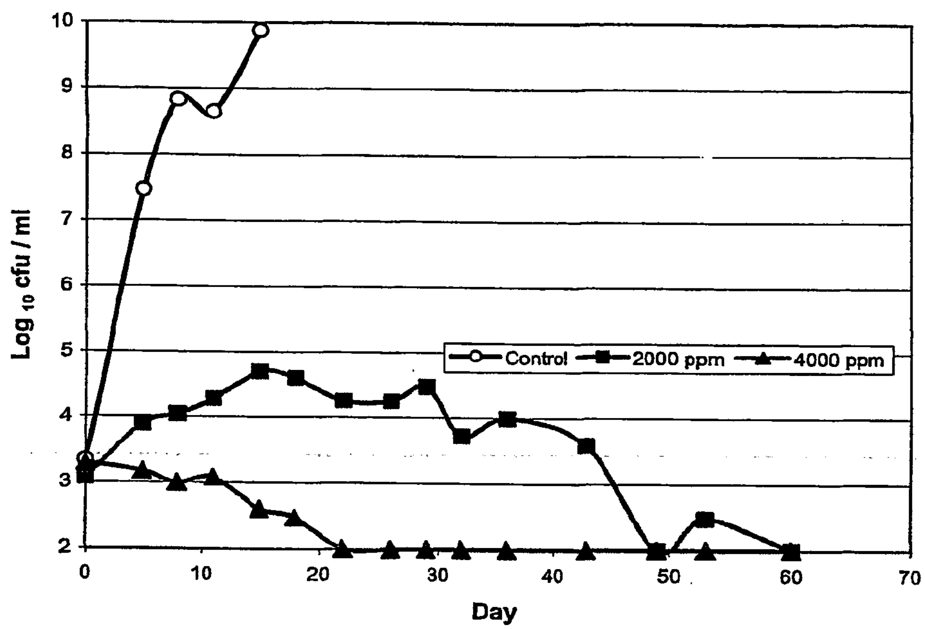
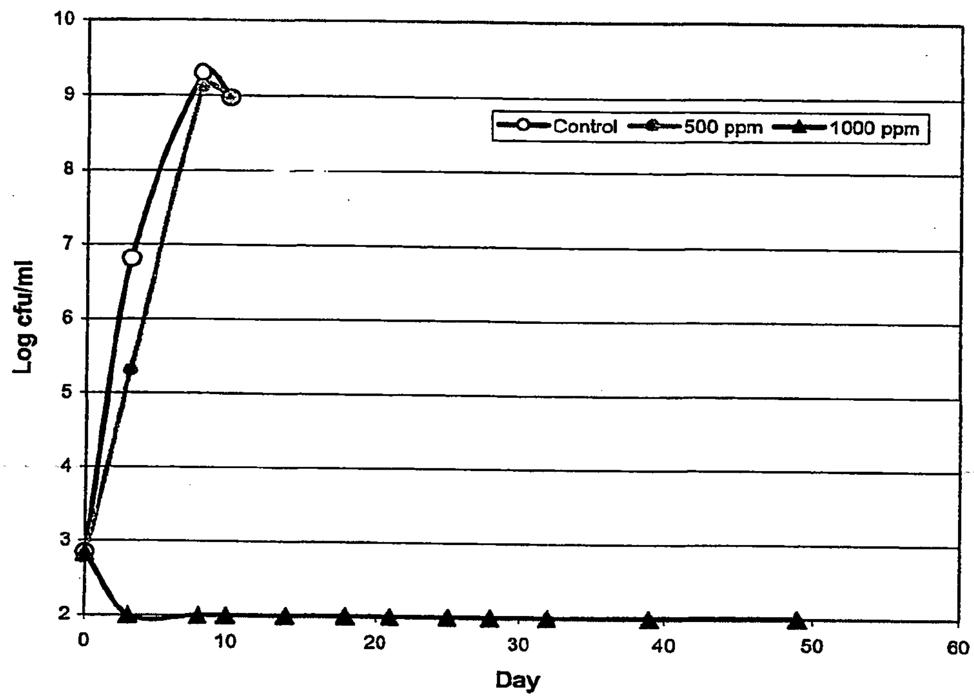

10
9
8
7
6
5
4
3
2
Log 10 cfu / ml
0
10
20
30
40
50
60
70
Day
Control
2000 ppm
4000 ppm

FIGURE 9


Control
500 ppm
1000 ppm
Log cfu/ml
Day
10
9
8
7
6
5
4
3
2
0
10
20
30
40
50
60

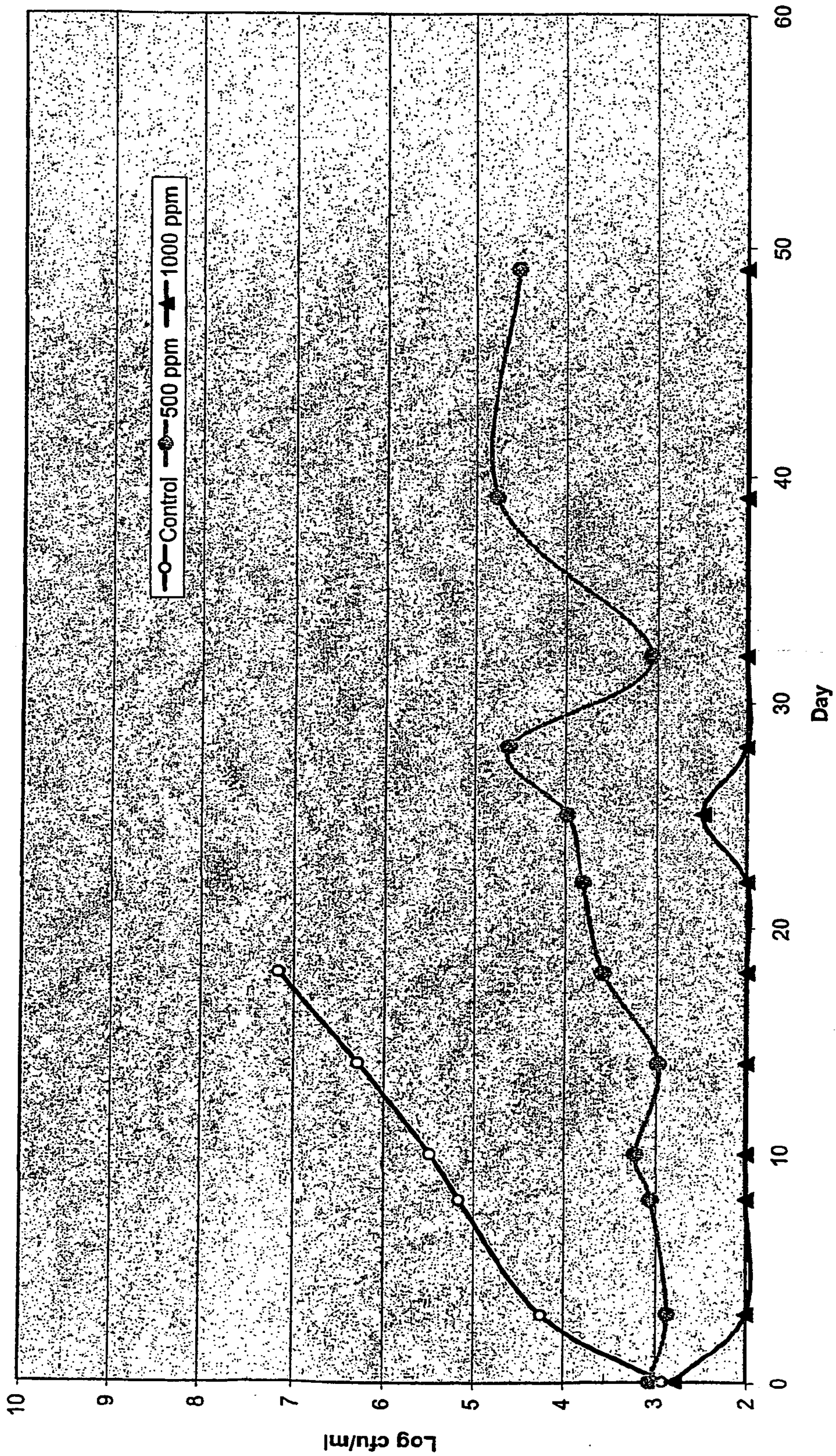


Figure 10

Figure 11

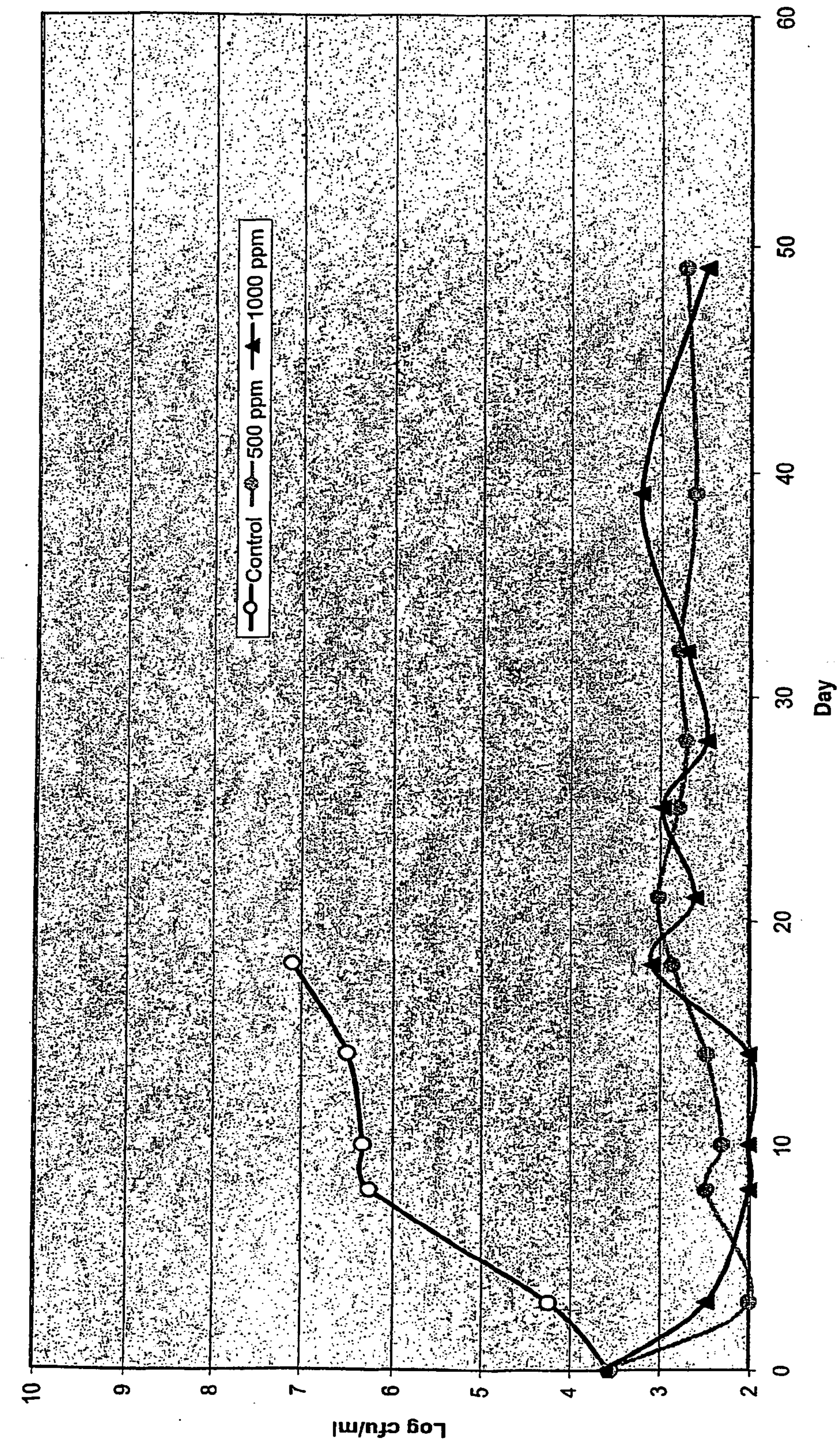
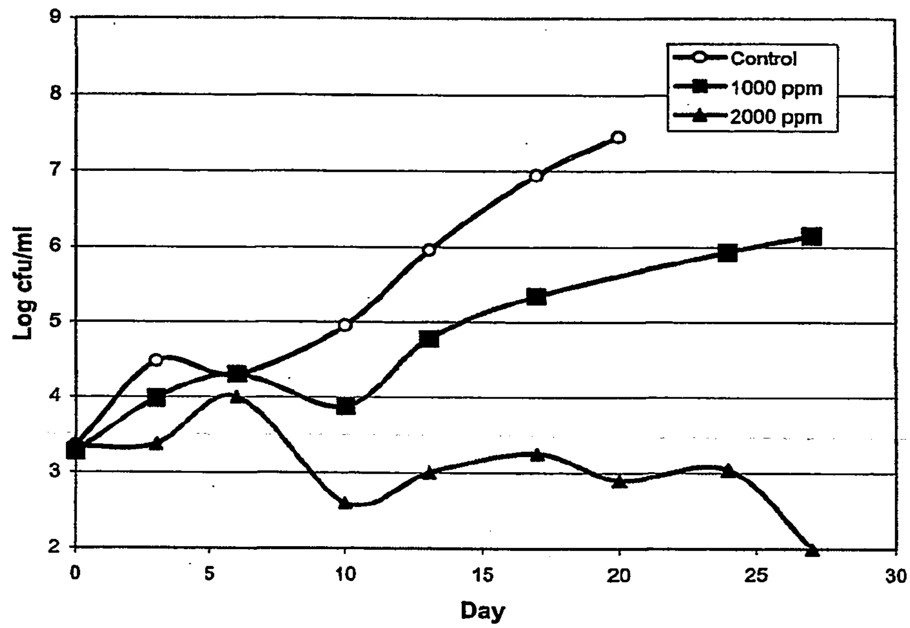
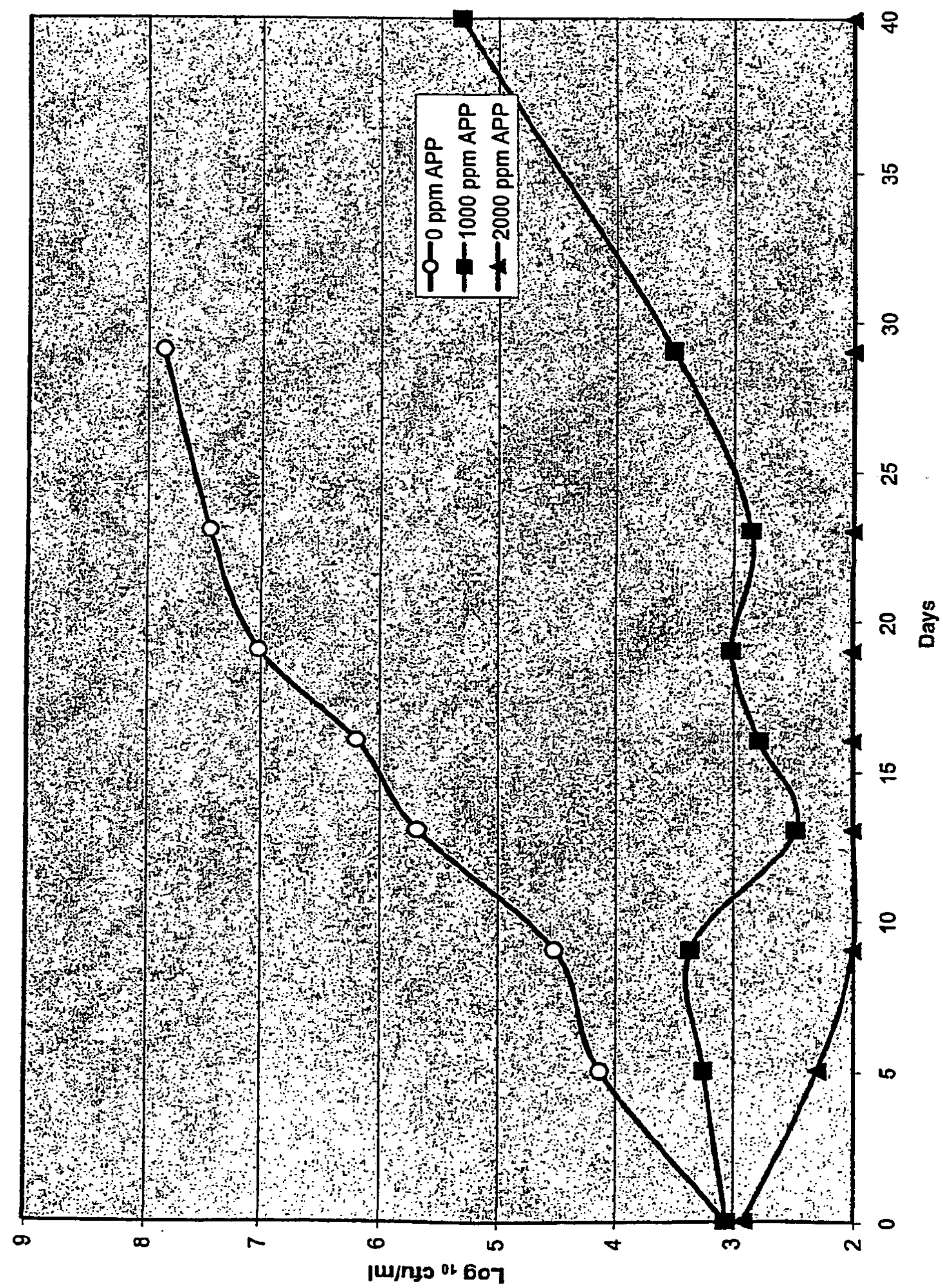

Control
500 ppm
1000 ppm
Log cfu/ml
10
9
8
7
6
5
4
3
2
Day
0
10
20
30
40
50
60

FIGURE 12



Control
1000 ppm
2000 ppm
Log cfu/ml
Day
9
8
7
6
5
4
3
2
0
5
10
15
20
25
30

Figure 13


0 ppm APP
1000 ppm APP
2000 ppm APP
Days
Log 10 cfu/ml
0
5
10
15
20
25
30
35
40
2
3
4
5
6
7
8
9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 01/04328

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23L3/3562 A23L3/3544 A61L2/16 A61L2/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23L A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

FSTA, EPO-Internal, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	DATABASE WPI Section Ch, Week 200138 Derwent Publications Ltd., London, GB; Class B03, AN 2001-360366 XP002186345 & JP 2001 089377 A (NIPPON DENPA KOGYO KK) , 3 April 2001 (2001-04-03) abstract ---	1-17
X	BAUTE M-A ET AL: "ENZYME ACTIVITY DEGRADING 1,4-ALPHA-D-GLUCANS TO ASCOPYRONES P AND T IN PEZIZALES AND TUBERALES" , PHYTOCHEMISTRY, PERGAMON PRESS, GB, VOL. 33, NR. 1, PAGE(S) 41-45 XP000925242 ISSN: 0031-9422	1-17
Y	page 43, column 2, paragraph 3 --- -/--	17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 January 2002	08/02/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Guyon, R

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 10616 A (DANISCO) 20 April 1995 (1995-04-20) claims 1,21,22; examples 4-1,4-2	17
A	WO 96 12026 A (DANISCO ;YU SHUKUN (SE); BOJSEN KIRSTEN (DK); MARCUSSEN JAN (DK)) 25 April 1996 (1996-04-25) page 9, line 5 -page 12, line 27	1-17
A,P	WO 01 56408 A (MUROYA KENKOU ;ASAMA KASEI KK (JP); FUJISUE MAMI (JP); NOZAKI KAZU) 9 August 2001 (2001-08-09) abstract figures 1-3	1
X,P	WO 00 56838 A (ANDERSEN SOEREN MOELLER ;JENSEN HENRIK MAX (DK); DANISCO (DK); ISA) 28 September 2000 (2000-09-28) page 1, line 5 - line 12; claims 1,4,11,12,14; figure 1; example 5 page 4, line 7 -page 5, line 19	1-17
X,P	WO 00 56745 A (ANDERSEN SOEREN MOELLER ;DANISCO (DK); MARCUSSEN JAN (DK); LUNDT I) 28 September 2000 (2000-09-28) page 11, line 7 - line 26; claims page 5, line 11 -page 9, line 25	1-17
A	GB 2 358 137 A (DANISCO) 18 July 2001 (2001-07-18) page 21, line 7-10; claims 1-12 page 13, line 9 -page 16, line 2	1-17
A	FR 2 617 502 A (ELF AQUITAINE) 6 January 1989 (1989-01-06) page 5, line 1 - line 8	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

al Application No
PCT/GB 01/04328

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			CA	2174115 A1	20-04-1995
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